Biochemical Methods in Cell Culture and Virology

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Preface

The procedures used in cell culture and virology are combined in this volume, because a study in either discipline may require an investigator to have a working knowledge in both to design meaningful experiments. While cultured cells now are used routinely by the virologist, it is important for the cell biologist to be aware that animal cells growing *in vitro* can sometimes carry endogeneous or defective viruses that modify the phenotypic expression of genetic information. For the student considering a career in virology, cell biology, or cancer research, it is hoped that this volume will serve as a useful guide for growing, handling, and studying viruses and cultured cells. Many of the procedures outlined have been developed since the author's first exposure to cell culture and virology some 20 years ago, and most have been used routinely or attempted at least once in the research and teaching laboratory here at Rutgers University in the past decade.

The book is divided into three parts, which are titled Cell Culture, Virology, and Macromolecular Analysis. The procedures used for culturing cells dispersed from tissues and organs are reasonably well established and are covered in Part I. These methods essentially all stem from the original observation that cells could grow attached to a glass surface in sheets from tissue fragments if the fragments were immobilized against the floor of the flask with perforated cellophane. Later, it was discovered that cells could be grown directly attached to glass without the cellophane covering, and that populations of viable cells for *in vitro* culture could be obtained by digesting tissue and organ fragments with the enzyme trypsin. These initial successes led to the development of adequate nutrient media for the growth of cells *in vitro* and to studies concerned with the passage of diploid and heteroploid cells.

Procedures for isolating, identifying, growing, and purifying viruses are covered in Part II. Although intact animal and embryonated egg procedures are described, major emphasis is placed on those methods in which cultured cells are used for

studying viruses. The impetus for using animal cells growing *in vitro* in virology came from the discovery that the poliovirus, long considered strictly as a neurotropic agent, could induce pathological changes in cells cultured from human embryonic organs. Other observations indicating that many other viruses could replicate in a variety of cell types from different animals, and that single infectious particles could form visible plaques on lawns of cultured cells, caused an immediate expansion in the use of this technology. When the efficacy of viral vaccines produced in cultured cells was established, novel methods for growing animal cells in mass culture were introduced.

Part III of this volume has been organized to include various biochemical and some biophysical methods that are used for studying deoxyribonucleic acids, ribonucleic acids, and proteins in viruses and cultured eucaryotic cells. The procedures covered in this section are fairly new, and are still being improved to achieve either an amplified or a more finely tuned result. Therefore, the procedure now used may differ in some detail from that presented here, and the modification will be found in the more recent research literature.

I would like to express my sincere gratitude to the following persons, who have introduced many of the procedures in my research laboratory and have described the various pitfalls that might be encountered: Dr. Robert Heflich, Dr. George Cukor, Dr. Howard Savage, Dr. Samuel Asculai, Dr. Claire Engle, Dr. Charles Bendas, Mr. Kenneth Soprano, Mrs. Nancy Hayner, Mrs. Donna Cryan, and Mrs. Erica Davis. A special note of thanks is extended to Dr. Wayne Umbreit, who encouraged me to begin this bidisciplinary work, and to Mrs. Elaine Derry, who skillfully typed the manuscript. Finally, an abiding appreciation is also extended to my wife, Martha, who provided advice and commentary during the many months of preparation of this work.

R.J.K.

Contents

I

Cell Culture

1

Culturing and Handling Cells *In Vitro*

The cells from embryonic, adult and tumor tissue of animals can be grown *in vitro*. The procedure followed in most laboratories is to free the cells from tissues and organs by mechanical, enzymatic, or chemical means. The dispersed cells are resuspended in an appropriate nutrient medium and inoculated into culture vessels, which are incubated in a stationary position. After settling out, the cells adhere to and stretch out on the surface, and assume a morphology close to that *in vivo*. In time the cells multiply until a confluent sheet is formed.

The best method for disaggregating a tissue or an organ into its constituent cells for *in vitro* culture is one that causes the least damage to the cells. The objective of any disaggregation procedure is to disrupt or digest intercellular materials that bond cells together, or hold them within an enmeshed tissue network. The gentlest procedure that might be used to disaggregate cells is one in which the bonding materials are all disrupted, so that the tissue literally falls apart into a population of single cells. With some organs it is possible to achieve this result by perfusion with an enzyme or a chemical. However, many organs are not easily perfused, and the technique almost always requires considerable skill. Therefore, the procedure used most often is simply to mince the tissue into small fragments with scissors, scalpels, razor blades, or cataract knives. To reduce cellular damage, it is important to use sharp instruments to avoid shearing (Rinaldini, 1958). Mechanical dispersion of the fragments into populations of cells is then possible with some tissues, especially those from young embryos. Such methods may involve the simple application of pressure in a homogenizer, passage through a nylon or stainless steel mesh, or the teasing of cells from the tissue fragments. With tissues taken from older animals and tumors, the intercellular bonds cannot be broken by mechanical means without shearing a large majority of the cells (Waymouth, 1974). To disaggregate the cells from these tissues, enzymes that digest the intercellular materials and chemicals that remove ions involved in stabilizing these materials are employed. A method

requiring nothing more drastic than brief exposure to a solution of pH higher or lower than the physiological level (Longmuir and apRees, 1956) or to a solution of abnormally high osmolality (McLimans, 1969) may suffice to separate cells from each other in a tissue fragment. Enzymes, such as trypsin, collagenase, pronase, elastase, hyaluronidase, papain, and deoxyribonuclease have been used to free cells from fragments of tissues and organs (Rinaldini, 1959). Chemical compounds, such as Ca^{2+}-free ethylenediaminetetraacetic acid (EDTA), ethylene glycol (2-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), citrate, and tetraphenylboron are used to bind cations, which are also involved in the intercellular bonding of cells in tissues and organs of animals (Zwilling, 1954; Rappaport and Howze, 1966a).

USE OF TRYPSIN FOR ORGAN AND TISSUE DISAGGREGATION

Acetone powders of bovine and porcine pancreas have been used for more than 30 years to disaggregate organs and tissues into populations of cells for *in vitro* culture. These commercially available powders are labeled as trypsin 1:250 or 1:300, because the activity of this enzyme is used to standardize the preparation. However, the nomenclature is unfortunate because these products contain elastase, chymotrypsin, and other enzymes as well as the endopeptidase trypsin. In recent years, highly purified crystalline trypsins have been used for disaggregating tissue fragments, but the crude enzyme preparation remains popular because its utility has been so convincingly demonstrated (Waymouth, 1974).

Rous and Jones (1916) first used a trypsin powder, which they partially purified to disperse cells growing out from tissue fragments explanted in plasma clots, and showed that the freed cells could be subcultured in fresh plasma. Vogelaar and Ehrlichman (1934) used an enzyme powder for subculturing primary cultures of thyroid tissue to determine if fibroblasts could continue to proliferate from explants that were repeatedly removed from plasma clots at 14-day intervals. But it was not until 1941 that Medawar demonstrated that a BDH (British Drug houses) trypsin powder could be used to separate pure epidermal epithelium uncontaminated by mesodermal elements from human skin. He attributed this to the fact that elastic fibers which anchor the epidermis to the underlying tissue are specifically dissolved by crude trypsin. Billingham and Medawar (1951) later concluded that the enzyme responsible for this effect was probably elastase.

Dulbecco (1952) then showed that pure trypsin could be used to separate whole chick embryos into populations of cells that could be directly cultured *in vitro* and used for virus titrations. Moscona (1952) demonstrated that the limb buds from chick embryos could be completely disaggregated with 3 percent trypsin (BDH powder) into populations of single cells if Ca^{2+} and Mg^{2+} salts were omitted from an otherwise balanced salt solution. The use of Ca^{2+} and Mg^{2+} free solutions is based on the evidence that these cations play a role in stabilizing the intercellular materials and the mutual adhesiveness of cells *in vivo* (Zeidman, 1947). When it was found that monkey kidney cells were susceptible to a variety of human

viruses, Youngner (1954) introduced a trypsinization procedure for disaggregating kidney fragments with the crude enzyme, so that large numbers of replicate cultures could be grown. Scherer et al. (1953) showed that trypsin could also be used for the continuous serial passage of established epithelial cells in culture. In their study, two enzyme preparations were employed, recrystallized trypsin (Armour Pharmaceutical Company, Chicago, Ill.) and trypsin 1:250 (Difco Laboratories, Detroit, Mich.). The recrystallized enzyme was not toxic for the established cells below 3 percent and a concentration of 0.5 percent was adequate for dispersing these cells. On the other hand, the crude enzyme was toxic at a concentration above 0.5 percent.

In 1958, Rinaldini observed that a highly purified trypsin was less effective than crude trypsin or other pancreatic enzyme preparations used for digesting intercellular materials, and emphsized that other enzyme activities which disaggregate tissue fragments are present in these preparations. Rinaldini (1959) examined the roles of pure trypsin, elastase, papain, and pancreatin on the disaggregation of chick heart tissues, and noted that crystalline trypsin, although useful for detaching cells from a glass surface, does not completely release cells from tissue, but leaves them enmeshed in a viscous gelatinous mass. The gel-like material, which traps the cells, makes handling very difficult. DNAse, pancreatin, elastase, and other crude trypsin preparations are able to digest this mucoidal material left after digestion with purified trypsin.

Crude trypsin preparations obtained from commercial suppliers vary in their relative ability to disaggregate tissue fragments into populations of free cells. Pine et al. (1969a, b) attempted to determine if the relative activities of elastase, trypsin, chymotrypsin, ribonuclease, lipase, collagenase, and phosphatase in different lots of crude trypsin were related to their effectiveness for disaggregating tissues. Although the results were inconclusive, it was found that a combination of crystalline trypsin, chymotrypsin, and elastase, modeled after the activities in good batches of crude trypsin, were more effective in disaggregating kidney fragments than the individual enzymes.

Prolonged immersion of cells, especially in crude trypsin, will damage them. To minimize this damage, the cells should be removed from the enzyme solution as soon as possible, or an inhibitor of the enzyme should be added to neutralize the activity. Serum can be added for this purpose at a concentration of 5 to 10 percent since it contains natural trypsin and elastase inhibitors. Crystalline trypsin, on the other hand, will digest dead cells but not living cells. The purified enzyme, however, does cause cellular modifications, which are mostly surface related. Some of these include (1) the liberation of surface glycoproteins, (2) the removal of surface antigen, (3) the alteration of electrophoretic mobility, and (4) the alteration of surface ultrastructure. These changes can eventually damage a cell if the exposure to the enzyme is extended for long periods of time. The question concerned with the uptake of trypsin by viable cells has been periodically raised to account for the damage caused after prolonged exposure. Using fluorescein-labeled and tritium-labeled crystalline trypsin, it has been found that the enzyme is at first localized on the surface, and later penetrates into the cell at 25 and 37°C, but not at

4°C. Intracellular localization persists for about 48 hours after the cells are removed from the trypsin solution. This result indicates that the enzyme may affect certain intrinsic properties of the cell (Hodges et al., 1973).

Preparation of Trypsin

Crude pancreatic preparations with tryptic activity can be purchased from commercial suppliers in various forms. Acetone powders are available that are designated trypsin 1:250 (Difco Laboratories, Detroit, Mich.) and 1:300 (General Biochemical Company, Cleveland, Ohio; Schwarz/Mann Laboratories, Orangeburg, N.Y.), which means that the enzyme will digest 250 or 300 parts of casein under the conditions of the U.S.P. test. Individual lots of trypsin will vary in their ability to disaggregate tissues, so that each should be tested before being used for this purpose (Pine et al., 1969b). Sterile, frozen trypsin solutions can be purchased from commercial suppliers of products for cell culture at concentrations of 0.25 percent (1X) and 2.5 percent (10X). Also available for dissaggregating tissues are trypsin preparations containing EDTA. A 1X solution contains 0.05 percent trypsin and 0.02 percent EDTA. About 20 percent of the trypsin activity is lost in the presence of EDTA, because Ca^{2+} is needed to stabilize the enzyme. The following procedure is used for the preparation of a trypsin solution from an acetone powder.

1. Two and one-half grams of trypsin powder (1:250 or 1:300) is placed into a large beaker. Five milliliters of calcium- and magnesium-free physiological balanced salt (CMF-PBS) (8.0 g of NaCl, 0.3 g of KCl, 0.73 g of Na_2HPO_4, 0.20 g of KH_2PO_4, and 2.0 g of glucose to 1000 ml with H_2O) is added, and the trypsin is made into a smooth paste.

2. Nine hundred milliliters of CMF-PBS is added with continuous stirring, which is essential for dissolution of the trypsin.

3. The mixture is made up to 1000 ml by adding CMF-PBS, and the small amount of residual material is removed by passing the solution through filter paper.

4. The pH is adjusted to 7.8 by the addition of 0.1 *N* NaOH. This pH is a good compromise between the optimum pH for tryptic activity (pH 8.2) and that which is physiological for cells (pH 7.2 to 7.4).

5. The solution is sterilized by filtration, dispensed in convenient volumes, and stored at -20°C. At 4°C The enzymatic activity is lost slowly over a 4-week period. For convenience, a 1-week supply of 1X trypsin can be kept in the refrigerator.

Organ and Tissue Disaggregation

Procedures for disaggregating kidney, chick embryo, and human amnion tissue fragments with crude trypsin into populations of cells for *in vitro* culture are presented. These procedures can be modified for isolating cells from other tissues and organs or tumors for *in vitro* culture.

Kidney disaggregation

The following procedure is similar to those described by Youngner (1954), Melnick (1955), and Rappaport (1956) for disaggregating monkey kidney tissue with crude trypsin preparations.

1. Organ removal and dissection: Monkeys weighing 5 to 7 lb (2.2 to 3.2 kg) are anesthetized with ether and exsanguinated by cardiac puncture. The kidneys are removed aseptically and placed into a sterile petri dish containing warm Hanks' balanced salt solution (BSS). Each kidney is then transferred to another sterile dry petri dish for further dissection. The capsule is removed and each kidney is cut in half horizontal to the flat surface. The cortex is separated from the medulla by holding the bisected organ with a forceps and cutting it with a scissors. Both the capsular and the medullary tissue are discarded.

2. Enzyme digestion: The cortical tissue is cut into 1-cm^2 pieces. These fragments are washed several times with BSS and transferred to a 500-ml trypsinization flask similar to the one shown (Melnick et al., 1955) (Figure 1–1), and covered to a level of 150 ml with the 0.25 percent trypsin in CMF-PBS warmed to 37°C. A 500-ml flask is large enough to trypsinize an initial load of 80 g of tissue. If less than 30 g of tissue is to be disaggregated, a 250-ml flask is used. The fragments in the flask are kept in motion by agitation on a rotary shaker or with a magnetic stirring device. The speed of agitation should be sufficient to allow rapid mixing with cavitation but no foaming. In either case, the temperature is controlled at 37°C.

3. Cell collection: After 1 hour, the agitation is stopped and the tissue fragments are allowed to settle. The fluid phase containing some free cells is decanted from the side arm into a sterile 500-ml erlenmeyer flask kept at 4°C. The trypsin-

Figure 1–1
Trypsinization flask. Courtesy Bellco Glass, Inc., Vineland, N.J.

izing flasks are designed with a specially constructed neck that protrudes inward and acts as a dam so that fragments are not poured off with the fluid phase.

4. Subsequent trypsinization cycles: Fresh, prewarmed trypsin is added to the flask to the same level, and the fragments are agitated for 7 minutes. At this point the tissue fragments are again permitted to settle, and the fluid phase is decanted through the side arm into the flask containing the first supernate. This step is repeated until the tissue is exhausted. The number of runs required varies with the mass of the tissue. A total of 8 cycles is usually sufficient for four kidneys, and 10 to 13 cycles for eight kidneys. At the end of each cycle, the remaining fragments are washed clean of any loosely attached cells by adding 50 to 100 ml of trypsin solution, swirling the flask, and decanting the fluid phase into the collection flask. After the third or fourth cycle, it may be necessary to wash the fragments two to four times before proceeding with the next cycle, because many of the loose cells are easily freed. The washing step should be done without haste, and may take 5 to 10 minutes. If the tissue has been disintegrated significantly during a cycle, the decanted fluid when held up to light will be seen as a fine suspension of tissue fragments and cells.

5. Removal of cells from the enzyme solution: The combined fluids in the collection flask are centrifuged at less than $50 \times g$ for 30 minutes. The supernatant fluid is removed from the packed cell mass to within 1 or 2 ml by using a vacuum aspirator. The procedure should yield appfoximately 3 ml of packed cell mass for each kidney trypsinized.

6. Culturing the freed cells: The packed cells are resuspended in about 100 ml of growth medium, prewarmed to 37°C. If a cold medium is used, the cells clump badly. The cell suspension is filtered through two layers of sterile cheese cloth. The gauze filter is washed free of adhering cells with about 100 ml of nutrient solution. The number of cells per milliliter of suspension is determined by counting in a hemocytometer or in an electronic cell counter. The preparation of eight kidneys takes about 4 to 5 hours. An average yield is between 6.0×10^7 and 8.0×10^8 cells per gram of cortical tissue. The cell suspension is diluted to 300,000 ml and distributed into culture tubes or flasks for attachment and growth. Melnick's medium A (p. 84) with 2 percent calf serum is often used for growing primary monkey kidney cells.

Continuous automatic trypsinization method. An apparatus for the continuous dispersion of cells from tissue fragments has been designed by Rappaport (1956). Essentially, the tissue fragments are placed into a specially constructed flask (Figure 1-2), and the flow of trypsin from a reservoir is controlled by a pinchcock. The fragments are agitated with a magnetic stirring bar. A sump containing numerous holes (1/2 to 1 mm in diameter) is annealed into the bottom of the flask. In this way only suspended cells can pass through the holes into the receiving vessel. The rate of outflow is controlled at the same rate as the trypsin inflow to allow the trypsin volume to remain constant. The trypsinization fluid containing freed cells flows into a bottle held in an ice-water bath. The cells are collected by centrifugation. More than 80 percent of the cells can be recovered from the renal cortex by the continuous procedure.

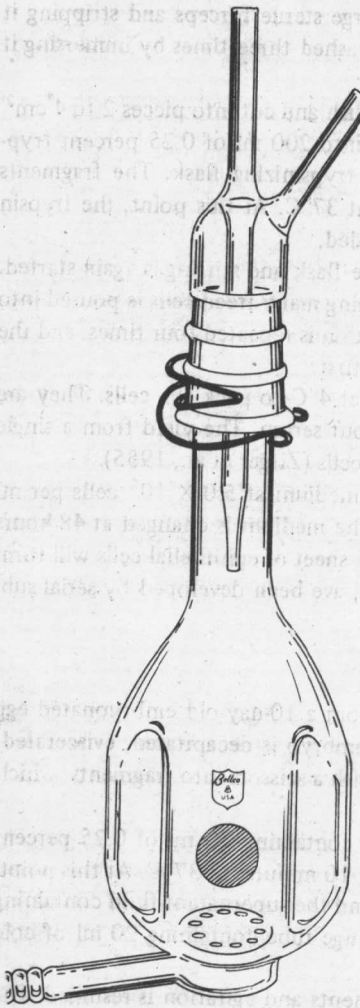

Figure 1-2
Continuous trypsinization flask. Courtesy Bellco Glass, Inc., Vineland, N.J.

Overnight trypsinization method. This procedure has been used for dissaggregating cells from monkey, hamster, bovine, canine, and rabbit kidneys.

1. Instead of incubating the tissue fragments at 37°C in step 2 of the kidney disaggregation procedure outlined above, the suspension is stirred, at a rate just short of bubbling, at 4°C overnight.

2. On the following day, the digest is decanted through a stainless steel wire cloth (72 mesh, wire diameter of 0.0037 in.; Newark Wire Cloth Company, Newark, N.J.) into a large centrifuge tube. The procedure starting at step 5 in the kidney disaggregation procedure is then followed.

Human amnion disaggregation

1. A normal full-term placenta is obtained in a sterile container. The side to which the cord is attached should face up. The amniotic membrane can be removed